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14. ABSTRACT

Mechanisms of Coagulation Abnormalities after Trauma Background. Trauma remains the leading cause of death and disability in patients under 40. Coagulopathy is common following trauma and is associated with poor outcome. Our group has identified an Acute Traumatic Coagulopathy, which this grant seeks to characterize. Objective/Hypothesis. Our preliminary human data indicate that there is a close correlation between the development of coagulopathy and the activation of the protein C pathway. Thus, in this work, we are testing the hypothesis that acute traumatic coagulopathy is primarily caused by tissue hypoperfusion resulting in a complement-mediated activation of the protein C pathway. Study Design. In the first objective, a single center, prospective cohort study is examining the timing and causes coagulation derangements after severe trauma and hypoperfusion. The second and third objectives continue to mechanistically define the role of the protein C pathway and complement in the development of these coagulation abnormalities. Relevance. During the first year of this grant we reported that that activation of the anticoagulant protein C is a critical mechanism driving early posttraumatic coagulopathy. Thus, further research into the mechanisms and treatment of coagulation dysfunction after trauma will continue to prevent early and late deaths in severely injured patients.

15. SUBJECT TERMS

Trauma, coagulation, inflammation

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1. Introduction

Trauma remains the leading cause of death and disability in patients less than 40 years old, eclipsing ischemic heart disease, stroke and HIV/AIDS. Coagulopathy is common following severe trauma and is associated with poor outcome. Classically, traumatic coagulopathy has been thought to be due to acidosis and hypothermic inhibition of clot formation as well as dilution of coagulation factors from intravenous fluid therapy. However, two recent studies have reported that one quarter of major trauma patients are coagulopathic upon arrival in the emergency department before any fluid resuscitation. This coagulopathy was dependent on concomitant hypoperfusion and hence unlikely to be exclusively due to tissue injury and coagulation factor consumption. Despite the understanding of a strong link between injury, hypoperfusion and bleeding, the mechanisms for hypoperfusion-induced coagulopathy after trauma are unknown.

Our preliminary human data indicate that there is a close correlation between the development of this coagulopathy and the activation of the protein C pathway early after severe trauma. Furthermore, the inhibition of the protein C pathway completely corrects that early posttraumatic coagulopathy in our mouse model of trauma-hemorrhage that mimics the clinical findings. Thus, in this application, we are testing the overall hypothesis that acute traumatic coagulopathy is primarily caused by tissue hypoperfusion resulting in a complement-mediated activation of the protein C pathway.

2. Body

Task 1. To determine the relationship between the activation of the protein C pathway, the complement cascade and the development of early coagulopathy and later end-organ injury associated with severe trauma in humans.

During the year 1 of the grant, we have performed a prospective cohort study of 203 major trauma patients admitted to a single trauma center. Serial blood samples were drawn at the arrival in the emergency department, 6 12 and 24 hours after admission to the hospital for analysis of partial thromboplastin and

prothrombin times, coagulation factors V and VIII activities, plasma levels of protein C, activated protein C, tissue plasminogen activator, and D-Dimers. Base deficit (BD) was used as a measure of tissue hypoperfusion.

A total of 203 trauma patients were enrolled. The results show that patients with tissue hypoperfusion and severe traumatic injury showed a strong activation of the protein C pathway in the bloodstream that was associated with a coagulopathy characterized by a deactivation of the coagulation factors V and VIII and a derepression of the fibrinolysis with high plasma levels of plasminogen activator and high D-dimers. Elevated plasma levels of activated protein C were significantly associated with increased mortality, organ injury, increased blood transfusion requirements, and reduced ICU ventilator-free days. Finally, the inability to recover physiologic plasma levels of protein C within 24 hours after trauma is associated with an increased risk to develop post-traumatic ventilator. The manuscript summarizing the results of this study is in press in the Annals of Surgery (1).

Task 2. To determine the role of protein C pathway in the development of early posttraumatic coagulopathy after trauma-hemorrhage in mice.

During the year 1 of the grant, we have performed a first experimental study to examine the mechanistic role of protein C in the development of early post-traumatic coagulopathy using a mouse model of trauma and hemorrhagic shock characterized by the combination of tissue injury and severe metabolic acidosis.

Mice were subjected to one of four treatment groups: 1) C- control, 2) T- trauma (laparotomy), 3) H- hemorrhage (MAP 35mmHg x 60 minutes), 4) TH- trauma + hemorrhage. After 60 minutes, blood was drawn for analysis. The results show that compared to C-mice, the TH-mice had a significantly elevated aPTT (23.3 vs 34.5 sec) and significantly increased levels of activated protein C (aPC) (2.30 vs. 13.58 ng/mL). In contrast, T- and H-mice did not develop an elevated aPTT or increased aPC. Selective inhibition of the anticoagulant property of aPC prevented the coagulopathy seen in response to trauma/hemorrhage (23.5 sec vs. 38.6 sec. [inhib. mAb vs control mAb]) with no impact on survival during the

shock period. However, complete blockade of both the anticoagulant and cytoprotective functions of aPC, caused 100% mortality within 45 minutes of shock, with histopathologic evidence of pulmonary thrombosis and perivascular hemorrhage. The manuscript summarizing the results of this study has been published in the Journal Shock (2).

Task 3. To determine the role of the complement in causing early coagulopathy following trauma-hemorrhage in mice.

During the year 1 of the grant, we have collected preliminary data in trauma patients indicating that the activation of complement within 30 minutes after severe injury correlates with the development of a protein C-mediated coagulopathy and fibrinolysis in trauma patients (**Figures 1-3**). These data included 200 severely traumatized patients from whom a blood sample has been taken immediately after the arrival to the hospital and before any fluid resuscitation. The data are represented as quartiles of each 50 patients (*$p <$ 0.05 from the lowest quartile).

Because of these clinical correlations, we then performed an experimental study in mice to examine the role of the complement mechanistic role of protein C in the development of early post-traumatic coagulopathy using a model of trauma and hemorrhagic shock characterized by the combination of tissue injury and severe metabolic acidosis that we have recently published (2). .We first found that the activation of the complement with cobra venom factor is associated with an increase in plasma levels of activated protein C (n=5 mice in each group, *$p <$ 0.05) (**Figure 4A**). Furthermore, we found that MBL null mice did not activate the protein C pathway after severe trauma and hemorrhagic shock (n=5 mice in each group, *$p <$ 0.05) (**Figure 4B**). These preliminary data indicate that the complement cascade is involved in the activation of the protein C pathway via its lectin pathway after trauma-hemorrhage. During the year 2 of the grant, we will continue to explore the mechanisms by which complement activates the protein C pathway. These experiments are important because they may provide new therapeutic approach to slow down the development of the protein C-mediated

early posttraumatic coagulopathy that is not responsive to the administration of coagulation factors with fresh frozen plasma.

Project Milestones

1. Six Months

Aim 1: Fifty patients enrolled. Milestone reached.

2. Twelve Months

Aim 1: Hundred patients enrolled. Milestone reached.

Aim 2: First series of mice experiments with the antibodies to aPC completed. Milestone reached.

3. Key research accomplishments

a. Demonstration that the early post-traumatic coagulopathy is mediated by the activation of the protein C pathway.

b. Demonstration that the inhibition of both the anticoagulant and cytoprotective functions of protein C is associated with poor outcome after trauma-hemorrhage, indicating an important protective role for the cytoprotective, PAR-1-dependent domain of the protein C in preventing systemic intravascular coagulation in the microcirculation after severe trauma-hemorrhage.

c. Demonstration that complement can activate the protein C pathway after severe trauma-hemorrhage via its lectin pathway.

4. Reportable outcomes

Published manuscripts that resulted from the work supported by the present award:

1. Cohen MJ, Call M, Rahn P, Calfee C, Esmon C, Brohi K, Baccheti P, Pittet JF. Critical Role of activated protein C in early coagulopathy and later organ failure, infection and death in trauma patients. Ann Surg (in press), 2011.

2. Chesebro BB, Rahn P, Carles M, Esmon CT, Xu J, Brohi K, Frith D, Pittet JF, Cohen MJ. Increase in activated protein C mediates acute traumatic

coagulopathy in mice. Shock 32:659-65, 2009.

5. Conclusion

Clinical study:

a. Early traumatic coagulopathy occurs only in the presence of tissue hypoperfusion and severe traumatic injury and is associated with the activation of the protein C pathway. Plasma levels of activated protein C at the admission to the hospital are predictive of clinical outcomes following major trauma. The inability to recover of physiologic plasma values of protein C within 24 hours after injury is associated with an increased propensity to later develop nosocomial lung infection, suggesting a role for the protein C pathway in the maintenance of the function of the alveolar-capillary barrier.

b. The activation of complement within 30 minutes after severe injury correlates with the development of a protein C-mediated coagulopathy and fibrinolysis in trauma patients.

Mice studies:

a. The results of the first mouse study indicate that our unique mouse model of trauma/hemorrhagic shock mimics our previous observations in trauma patients and demonstrates that early traumatic coagulopathy is mediated by the activation of the protein C pathway. In addition, the cytoprotective effect of protein C activation appears to be necessary for survival from the initial shock tissue injury.

b. The preliminary results of the second mouse study indicate complement can activate protein C via its lectin pathway.

So What Section: We have discovered a major new mechanism, the activation of the anticoagulant protein C pathway, that causes severe coagulation abnormalities after trauma-hemorrhage in humans. This discovery may lead to the development of new therapeutic approaches to correct the early coagulopathy that is associated with poor outcome after severe trauma in humans.

6. References

1. Cohen MJ, Call M, Rahn P, Calfee C, Esmon C, Brohi K, Baccheti P, Pittet JF. Critical Role of activated protein C in early coagulopathy and later organ failure, infection and death in trauma patients. Ann Surg (in press), 2011.

2. Chesebro BB, Rahn P, Carles M, Esmon CT, Xu J, Brohi K, Frith D, Pittet JF, Cohen MJ. Increase in activated protein C mediates acute traumatic coagulopathy in mice. Shock 32:659-65, 2009.

Critical Role of Activated Protein C in Early Coagulopathy and later Organ Failure,

Infection and Death in Trauma Patients

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Running Head: Acute traumatic coagulopathy is mediated by activation of protein C after shock and tissue injury.

Introduction

Trauma remains the leading cause of death and disability between the ages of 1 and 44, eclipsing ischemic heart disease, cerebrovascular disease and HIV/AIDS.[1] Worldwide, one in seven deaths is due to injury, and this is expected to rise to 1 in 5 in the next 15 years, despite continuing advances in resuscitation, trauma surgery, and critical care. Hemorrhage is the major mechanism responsible for death during the first 24 hours after trauma, and efforts to control hemorrhage and restore circulatory homeostasis form the core of the early therapeutic approach to traumatic injuries[2-5].

Perturbations in blood coagulation are common following major trauma and are associated with poor outcomes [6, 7]. Classically, coagulopathy associated with trauma is thought to be due to the consumption of coagulation factors, dilution from intravenous blood and fluid therapy, or hypothermia [8]. The traditional post-injury resuscitative protocol involves large volumes of relatively "cold" fluid (dilution), exposure of the patient (hypothermia), and prolonged surgery (more exposure, hypothermia and continued bleeding), all of which precipitate metabolic failure (acidosis) [2, 5]. These abnormalities have been characterized in animal models and clinical human research. There is also an extensive literature exploring the ideal resuscitative protocol and treatment of this iatrogenic coagulopathy [2, 8]. Consistent with this concept, recent retrospective studies from the Iraq conflict and from civilian trauma centers have shown that an increased plasma: red cell ratio (> 1:2) is associated with decreased mortality and improved long term outcomes in massively transfused trauma patients [9]. These results underscore the practical importance of coagulopathy after trauma.

It has recently been recognized that approximately one quarter of the severely traumatized patients present with a coagulopathy on arrival in the emergency department that is

physiologically and mechanistically distinct from the classical iatrogenic posttraumatic coagulopathy. Two studies have described that this acute traumatic coagulopathy is associated with higher transfusion requirements, a greater incidence of Multiple Organ Dysfunciton Syndrome (MODS), longer ICU and hospital stays, and a four-fold increase in mortality in coagulopathic patients compared to those with normal coagulation[10]. In two recent clinical studies, we have further characterized this early traumatic coagulopathy and have reported that when traumatic injury is combined with tissue hypoperfusion (shock), the resultant coagulopathy is characterized by a significant decrease in plasma protein C (PC) levels and an derepression of fibrinolysis [11-13]. Whether this early coagulopathy and protein C decrease is associated with the activation of protein C pathway has not been demonstrated and constitutes the first aim of this study.

Along with its anticoagulant effect, activated protein C has anti-inflammatory and anti-apoptotic properties that stabilize endothelial barrier function through its binding to PAR-1 and EPCR. Several studies have shown a beneficial effect of aPC on organ injury and mortality in both experimental and clinical investigations[14-17]. Indeed recombinant activated protein C (Drotrecogin alfa) remains the only FDA approved drug for the treatment of sepsis. It is thought that activated protein C protects from end organ damage in sepsis due to a combination of its anticoagulant effect preventing end-organ microvascular thrombosis and its direct cytoprotective anti-inflammatory effects. The reduction in plasma levels of activated protein C observed and putatively contributing to severe sepsis is thought to be the result of increased degradation, impaired production and reduced activation at the cell surface due to reductions in the expression of thrombomodulin (TM) and endothelial protein C receptor (EPCR) from transcriptional downregulation and cleavage. Indeed in severe sepsis the protein C pathway becomes

dysfunctional contributing to endothelial and epithelial cell dysfunction in this syndrome. Whether similar changes in the protein C pathway follow severe trauma is still unknown. We therefore hypothesized that the early activation of the protein C pathway in patients with severe trauma might be followed by a depletion of plasma levels of protein C much earlier than currently believed. Hence, the second aim of the study is to determine whether there is an association between activated protein C (aPC)-mediated acute traumatic coagulopathy and later propensity to infection, organ failure and mortality after severe trauma in humans.

Methods

The Institutional Review Board of the University of California at San Francisco approved the research protocol for this prospective cohort study and granted a waiver of consent for the blood sampling as a minimal risk intervention.

Patients

Consecutive major trauma patients admitted to the San Francisco General Hospital (level 1 trauma center) were studied. All adult patients who met criteria for highest-level trauma team activation by physiologic and anatomic triage criteria were eligible for enrollment. Patients were excluded if they were less than 18 years old, were prisoners, were pregnant or were transferred from other hospitals or had significant resuscitation prior to trauma activation. Patients were retrospectively excluded if they were later found to be on anticoagulant medications or possessing a preexisting bleeding diathesis.

Sample collection and measurements

Our sampling protocol and methodology has been described previously in detail.[12] Briefly, serial 10 ml samples of blood were drawn in citrated tubes upon arrival in the emergency department, 6, 12 and 24 hours after admission to the hospital. The samples were immediately transferred to the central laboratory, centrifuged and the plasma extracted and stored at -80°C. Samples were analyzed at the conclusion of the study by researchers who were blinded to all patients' data. Factors Va, VIIIa (activity), Protein C activity (PC), tissue plasminogen activator (t-PA), and D-Dimers were measured with a Stago Compact (Diagnostica Stago Inc., Parsippany, NJ). All tests were performed in accordance with the manufacturer's instructions. Activated protein C

measurements were performed from blood collected in tubes containing benzamidine, using an enzyme capture assay that we have successfully established in our laboratory.

Data collection, outcome measures

Data were collected prospectively on patient demographics, the injury time, mechanism (blunt or penetrating) and severity, pre-hospital fluid administration, time of arrival in the trauma room and admission vital signs. The Injury Severity Score (ISS) was used as a measure of the degree of tissue injury[18]. An arterial blood gas was drawn at the same time as the research sample as part of the standard management of major trauma patients. The base deficit was used as a measure of the degree of tissue hypoperfusion. Admission base deficit is a clinically useful early marker of tissue hypoperfusion in trauma patients, and an admission base deficit greater than 6 mmol/l has previously been identified as predictive of worse outcome in trauma patients[19, 20]. Ventilator-associated pneumonia (VAP) was diagnosed by blinded investigators from daily laboratory measurements, culture results, and daily chest x-rays which were obtained as standard of care at SFGH. Diagnosis was made by Centers for Disease Control and Prevention criteria[21, 22].

Outcome measures: Patients were followed until hospital discharge or death. For mortality analysis, patients surviving to hospital discharge were assumed to still be alive. Secondary outcome measures were also recorded for 28-day ventilator-free days, acute lung injury (American-European consensus conference definition)[21], VAP and acute renal injury (Acute Dialysis Quality Initiative consensus conference definition)[23] and blood transfusions required in the first 24 hours.

Statistical analysis

Data analysis was performed by the investigators and the Department of Biostatistics at UCSF. Normal-quantile plots were used to test for normal distribution. Relationships between injury quartiles and quartiles of activated protein C and continuous variables were tested with the Kruskall-Wallis test followed by a non-parametric test for trend. Correlation was assessed by Spearman correlation coefficients. Linear regression was used to test the relationship between injury and protein C and aPC and then between protein C and coagulopathy and continuous outcomes. Logistic regression was used to examine the relationship between the protein C system and dichotomous outcomes. A *p*-value of ≤ 0.05 was chosen to represent statistical significance. To define depletion of protein C after injury a slope of change was calculated between 0 and 12 hours and again between 12 and 24 hours. Depleters were defined as having a slope more negative than the median during both time periods. Balanced depleters had a positive slope in both time periods and depletion with recovery was defined as a negative slope between 0-12 hours and a positve slope between 12 and 24 hours.

Results

The trauma patients included in the study were severely injured with an average Injury Severity Score of 25$\pm$13 and average Base Deficit of -7$\pm$ 1. **Table 1** summarizes the type of injury and the demographics of the patients. As defined by an INR>1.3, 19.3% of patients were coagulopathic after injury.

Based on previous work and accepted definitions of tissue injury and shock (BD) (ref), we divided our patients into four equal groups based on ISS and base deficit. **Figure 1** shows that the combination of severe injury severity (ISS>15) and shock (BD>6) resulted in an increased PT (p=.01) and PTT (p=.03) upon arrival to the emergency department. **Figure 2** indicates that there was a strong activation of the protein C pathway only in patients who presented with the combination of severe tissue injury and shock. Plasma levels of aPC were elevated while plasma levels of PC were low in the trauma patients with shock and elevated ISS compared with the other groups of patients (Figure 2A&B p<.0001). There was a significant inverse correlation between initial plasma levels of PC and aPC (r = .313 p <.0001). Figure 2C&D show a significant relationship between increasing aPC and PT (p=.007) and aPC and PTT (p = .002). As expected, there was also a significant correlation between aPC and PT (r = .31 p =.003) and PTT (r =.287 p=.006) and an inverse correlation between PC and PT (r = -.39 p<.0001) and PTT (r =-.16 p =.05).

Exploring further the correlative mechanisms for this coagulopathy, **Figure 3** shows the relationship between activated protein C and coagulation factors and fibrinolysis. Figure 3 A&B show an inverse relationship between protein C activation and deactivation of factors Va (3A p = <.0001) and VIIIa (3B p = <.04). There is also an

direct relationship between aPC and fibrinolysis. Figure 3C&D shows that increasing levels of aPC are associated with increased tPA (p = .005) and D-Dimers (p =<.0001). Spearman correlation showed a significant inverse relationship between aPC and Factor Va (r=-.34 p = .001) aPC and factor VIIIa (r=-.21 p= .05) and a positive relationship between aPC and tPA (r=.41 p=.008) and aPC and d-Dimers (r= .58 p = <.0001).

Linear regression analysis confirmed a significant relationship between aPC and coagulopathy. Specifically, for every log increase in aPC there is a .57 increase in INR (95% CI 0.18-0.97; p= .005) and a 2.4 second increase in PT (95% CI 0.87-3.9; p =.005). This same relationship exists in reverse for non-activated protein C, where for each log decrease in PC there is a 1.2 increase in INR (95% CI 0.3 – 2.1; p =.01) and a 3.3 second increase in PTT (95% CI 0.04-6.7; p =.04). Taken together, these results demonstrate that severe trauma and hemorrhagic shock are associate with a strong activation of the protein C pathway that correlates with a coagulopathy characterized by a deactivation of the coagulation factors Va and VIIIa and a derepression of the fibrinolysis. Because we have previously reported that blocking the anticoagulant domain of aPC corrects the coagulopathy in a mouse model of trauma-hemorrhage with elevated plasma levels of aPC [24], it is likely that the activation of the protein C pathway mediates the early coagulopathy observed in trauma patients.

The second aim of the study was to determine whether there was a relationship between early activation of the protein C system and outcome from severe trauma. Because there is variability in baseline non-activated protein C levels, we used aPC to PC ratio to reflect the degree of activation of the protein C pathway at the time of

admission to the hospital. **Table 2** shows a strong association between activation of protein C and transfusion requirements. Protein C activation was further associated with an increased odds of ventilator associated pneumonia (OR 2.4, 95% CI 1.06-2.4; P=.02), an 1.6 fold increased odds of multiple organ failure (95% CI 1.0-2.5; p= .05), a 1.9 fold increased odds of acute lung injury (95% CI 1.2-3.1; p=.01) and an 2.1 fold increase in the odds of mortality (95% CI 1.3-2.3; p=.0007) (**Table 3**). In addition,patients with activation of protein C also spent significantly longer on the ventilator and had longer ICU and hospital stays (**Table 4).**

In the last series of analyses, we sought to determine whether the trauma-related activation of the protein C pathway was followed by the recovery of physiological plasma levels of protein C. We thus measured plasma levels of protein C at 0, 12 and 24 hours after admission to the hospital. We then calculated the individual and median slopes between plasma protein C values measured at these different time points. We finally divided our cohort of patients into 3 groups based on whether changes over time in their plasma levels of protein C were above or below the median values of these slopes. We found that the patient group with the greatest depletion in the first 12 hours followed by no recovery or further depletion from 12-24 (most negative slope) had an 2.7 times higher risk of VAP than the patients who did not change their plasma levels of protein C during the first 24 hours after admission to the hospital (95% CI 1.05-6.8; P= .03)**. (Table/Figure 4)** Taken together, these results indicate that the activation of the protein C pathway early after severe injury and hemorrhagic shock is associated with worse outcome in trauma patients. Furthermore, the inability to recover physiologic

plasma levels of protein C wthin 24 hours after trauma is associated with an increased

risk to develop VAP.

Discussion

We present the first evidence that the development of acute traumatic coagulopathy correlates with the activation of protein C pathway. We further report that after this initial activation of protein C, there is in some patients a rapid depletion of the plasma levels of the protein C zymogen that is associated with a significantly increased risk for developing nosocomial lung infection. Indeed maintenance of adequate levels of the protective protein C system prevents against later infection. These results represent a biological link between coagulopathic bleeding after injury and later infectious complications in the same trauma patients.

Coagulopathy after trauma is a common, and some degree of coagulopathic perturbation affects most severely injured patients at some point during their surgical and resuscitative course. For decades, it was commonly believed that any hypocoagulable state after trauma was due to iatrogenic mechanisms. Indeed, the accepted concept within the trauma community had long been that any coagulopathy after injury was secondary to hypothermia, dilution and metabolic acidosis that resulted from resuscitation and surgical care. Anecdotally however, several investigators had observed coagulopathy that took place before significant resuscitation, dilution and hypothermia. These observations were finally systematically studied in 2003 by Brohi and colleagues who documented in a large prospective study an acute traumatic coagulopathy 1 hour after injury in approximately 30% of patients[10, 11]. Patients arriving coagulopathic to the hospital had a significantly increased mortality of 40%. To test potential mechanisms to explain this coagulopathy, we performed a prospective study of 209 severely injured patients admitted to San Francisco General Hospital and reported

that this acute traumatic coagulopathy was associated with a depletion of plasma levels

of protein C zymogen at the admission to the hospital[12]. Subsequent work showed that

this effect was also present in patients with isolated traumatic brain injury[13]. However,

we did not measure in that study plasma levels of activated protein C. Furthermore, we

did not collect longitudinal sampling and we therefore were unable to examine the

relationship between early activation, later depletion of the protein C pathway and

subsequent propensity to organ failure or infection. Our new data shows that patients

with a combination of severe tissue injury (elevated ISS) and shock (elevated BD) are

coagulopathic nearly immediately after their injury. This coagulopathy is strongly

associated with the activation of protein C pathway. Further supporting protein C

activation are the strong inverse correlation between plasma levels of aPC and factor

Va and VIIIa inactivation and the derepression of fibrinolysis. Indeed, it is well known

that protein C, once activated through a thrombin-dependent reaction with

thrombomodulin (TM) and the endothelial protein C receptor (EPCR)[25], exerts profound

anticoagulant effects by irreversibly inactivating factors Va and VIIIa.[25, 26] This reaction

is augmented by the cofactor protein S, and serves to limit continued thrombin

production. In addition to its direct inhibition of fibrin formation, aPC also has an

anticoagulant activity mediated through the de-repression of fibrinolysis. Activated PC

directly inhibits plasminogen activator inhibitor 1 (PAI-1), which usually serves to limit t-

PA activity. Without the limitation of PAI-1, tPA is free to enhance the conversion of

plasminogen to plasmin and thereby enhance fibrinolysis. Hence aPC exerts its

profound anticoagulant activity by inhibiting coagulation <u>and</u> through derepression of

fibrinolysis[25]. As expected this activation of protein C and resultant coagulopathy is

associated with increased fluid and blood product resuscitation and poorer outcome (MOF, VAP and Death).

The second significant finding our study is the relationship between early coagulopathy, subsequent depletion of the protein C system and propensity toward infection. Indeed, our new data indicates that, as early as 6 hours after trauma (and initial coagulopathy), patients begin to segregate into those who are 'depleters' defined by depletion of protein C stores and those who maintain physiologic plasma levels of protein C zymogen. Those who deplete and do not recover their protein C plasma levels have a significant propensity to later infectious complications.

Along with its well-described anticoagulant functions, activated protein C also has newly described cytoprotective effects. Recombinant aPC has been shown to protect baboons and mice from sepsis and to attenuate LPS-mediated inflammatory signaling in monocytes [14, 15]. This non-anticoagulant effect of protein C is thought to be mediated through PAR-1 and EPCR, and multiple downstream signaling pathways including Rac-1 and NF-kappaB. Evidence for a link between protein C depletion and sepsis in humans has been described. Indeed, a well-established correlation between diminished plasma levels of protein C and worsened outcome exists in patients with septic shock.[27] In sepsis, protein C is directly cleaved and degraded by neutrophil elastase and circulating metalloproteases.[25, 27] Protein C activation is further diminished by protease cleavage of thrombomodulin (TM) as well as by a direct transcriptional down regulation of TM by circulating TNF-α and IL-6.[28] Protein C stores are also depleted by an

accelerated conversion to activated protein C (aPC), degradation by protein C inhibitor (PCI), and impaired hepatic biosynthesis, all of which contribute to a state of protein C and aPC depletion in sepsis.[25] Mesters has reported that the depletion of protein C (and reduction of activated protein C plasma levels) precedes the onset of any sepsis by an average of 12 hours. Other groups have reported diminished protein C levels in >90% of septic patients.[29-31] The end-result of protein C depletion and dysfunction is an increase in microvascular clot formation and in vascular endothelial permeability. Mechanistically, the contribution of microvascular thrombosis and endothelial leakiness to end-organ pathophysiology is also well described. As early as 1996, Asaka described thrombus formation in the liver of rats 5 minutes after endotoxin administration. With continued stimulation from LPS, areas of focal necrosis appeared secondary to local hypoperfusion and fibrin clot formation. Other groups have confirmed these findings in the gut, liver, and lung. Furthermore, skin biopsies from human patients with purpura fulminans that is associated with diminished plasma levels of protein C zymogen, show extensive microvascular thrombosis.[32] The understanding that a dysfunction of the protein C pathway is a likely mechanism for the end-organ damage observed after sepsis led to several groups testing the efficacy protein C, sTM, EPCR and activated protein C as therapy in septic animals and humans.[28, 33] The PROWESS study demonstrated a 6.1% reduction in mortality and a 19.4% reduction in 28-day mortality in septic patients who received recombinant aPC in continuous infusion for 96 hours[16].

Along with abundant data that septic shock is associated with protein C depletion, there is also evidence linking other shock states with protein C activation and depletion. Adrie

et al studied survivors of cardiac arrest and showed that patients in shock from cardiac arrest had increased plasma levels of aPC followed by later depletion to undetectable levels. [34] Along with increased plasma levels of aPC, these patients also were hypocoagulable early after cardiac arrest. Because perturbations in the activity of the protein C pathway are present in both septic and cardiogenic shock, it is not surprising that trauma and hemorrhagic shock would be associated with similar abnormalities in the protein C pathway.

There are several limitations in this study. First because we cannot obtain a baseline (pre injury) sample it is impossible to know the true level of activation for an individual patient. We attempted to control for this by using the aPC /PC ratio however we are aware that this is a best case approximation of true activation for each patient. Secondly knowing the true depletion of true activated protein C protective ability is also impossible. We hypothesize that the propensity towards infection in the protein C depletion group results from the inability to activate the non activated protein C in response to an infectious nidus or inflammatory stimuli however we are unable to

In conclusion, we present here the first evidence for an early activation of the protein C pathway that is associated with the acute coagulopathy observed in severely traumatized patients. In addition, we found a biological link between this early coagulopathy, later depletion of protein C stores and propensity to develop nosocomial lung infection, a common complication in severely injured patients who survive their initial injury. The, combination of the present clinical data with our previously published

data on the mechanistic role of the protein C pathway in the development of coagulopathy associated with trauma-hemorrhage in mice [24] indicate that the anticoagulant function of activated protein C represents an unfortunate side-effect of a profound anti-inflammatory response being released by the body in response to severe trauma and shock. In our previous mouse work, the cytoprotective effect of aPC was necessary for survival through the acute phase while the anticoagulant function when blocked had no effect on survival. Taken together with these data it seems that acute traumatic coagulopathy represents a maladaptive response to severe injury. Indeed, after severe trauma and tissue hypoperfusion, the body is likely releasing a large amount of an antiinflammaroy and cytoprotective molecule (aPC) in an attempt to prevent the development of a lethal microvascular thrombosis and endothelial and epithelial destruction. In a perfect example of maladaptive response, activation of the protein C pathway has profound anticoagulant effects which has the unfortunate (after severe injury) sequalie of coagulopathy. Shortly after this 'to much of a good thing' response which is complicated by a bleeding diathesis, there is In certain trauma patients, a depletion of the response ('to little of a good thing later') and a propensity to orgain injury and infection. The implications of this connection between early acute traumatic coagulopathy and later organ dysfunction and propensity to infection are significant. Indeed, future studies are warranted to identify drivers of both the early coagulopathy and later depletion of the protein C system. From this knowledge, putative future clinical intervention could involve blocking of the anticoagulant domain of aPC early after trauma, which would correct the early posttraumatic coagulopathy, while maintaining the cytoprotective effect of that protein that is critical for the homeostasis of

the vascular endothelium. Later, augmentation of the depleted protein C response by the administration of a protein C mutant that does not have the anticoagulant effect of the wild-type protein could be considered. Indeed, this mutant aPC form exists and has been shown to protect the vascular endothelium in several experimental models of ischemia-reperfusion and to decrease the severity of lung injury induced by *P. aeruginosa* in a mouse model of pneumonia (JF Pittet, personal communication). There is thus a need for additional experimental and clinical studies to fully understand the role the protein C pathway after severe trauma.

Figure Legends

Figure 1: A combination of tissue injury and shock result in coagulopathy in trauma patients. Patients were divided into groups using previously described definitions of injury severity based on Injury Severity Score and Shock based on Base Deficit. This resulted in 4 groups; minimal injury, no shock (ISS<15 BD<6); minimal injury, shock (ISS>15 BD >6); severe injury, no shock (ISS>15 BD<6); and severe injury and shock (ISS >15, BD >6). Prothrombin time (PT) and partial thromboplastin time (PTT) were assayed. Patients with severe injury and shock had elevated PT (Panel A) and PTT (Panel B). All p<.05 by Kruskall Wallis rank test.

Figure 2: Tissue injury and shock result in a systemic activation of protein C pathway associated with coagulopathy in trauma patients. Patients were divided into groups using previously described definitions of injury severity based on Injury Severity Score and Shock based on Base Deficit. This resulted in 4 groups; minimal injury, no shock (ISS<15 BD<6); minimal injury, shock (ISS>15 BD >6); severe injury, no shock (ISS>15 BD<6); and severe injury and shock (ISS >15, BD >6). Plasma levels of activated protein C (aPC) and protein C (PC) were assayed, as described in the Methods. Patients with severe injury and shock had elevation of plasma levels of aPC (Panel A) and a concomitant decrease in levels of PC (Panel B). aPC levels were then divided into quartiles. Patients with the highest quartile of plasma levels of aPC had elevated PT (Panel C) and PTT (Panel D). All p<.05 by Kruskall Wallis rank test.

Figure 3: Systemic Activation of the protein C pathway is associated with the inactivation of factors Va and VIIIa and derepression of fibrinolysis in trauma patients. Patients were divided into quartiles based on plasma levels of activated protein C. Patients with the highest activation of protein C in the plasma (highest quartile) had a coagulopathy caused by deactivation of factor Va (Panel A) and factor VIIIa (Panel B). In addition, patients with the highest plasma level of aPC had a derepression of the fibrinolysis, as evidenced by elevated tPA and d-Dimers (Panel C&D).

Figure 4: Protein C depletion is associated with an increased incidence of nosocomial lung infection in trauma patients. Patients were divided in three groups based on changes in plasma levels of protein C during the first 24h after trauma. In one group of patients there was a significant increase in the plasma levels of protein C zymogen within 12 hours after trauma. The rest of the patients who had a decrease in the plasma levels of proten C zymogen were divided into two groups characterized either by a moderate decrease of plasma levels of PC with recovery or by a significant decrease in plasma levels of PC without recovery during the first 24 hours after trauma. Patients who decreased their plasma levels of PC without any recovery had a 2.7 times increase in nosocomial lung infection than those we show some recovery of the plasma levels of PC between 12 and 24 hours after trauma (OR 2.7 CI 1.05 -6.8 p = .04).

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Table 1: Injury and Demographics

VARIABLE	Mean	SD
AGE	41.0	18.8
Gender Male	79%	
Mechanism Blunt	62%	
Mechanism Penetrating	38%	
ISS	25.2	13.8
Base Deficit	7.0	5.6
Pre sampling Resuscitation	217cc	319cc

ISS: Injury severity score

Table 2: Linear regression testing the effect of activation of protein C on transfusion and resuscitation requirements.

Predictor	Dependent	B	OR	p value
aPC/PC ratio	6h PRBC (Units)	6.7	2.5 - 20.9	.003
aPC/PC ratio	6h FFP (Units)	5.3	2.4 - 8.1	<.0001
aPC/PC ratio	6h Platelets (Units)	.57	.15 - .99	.009
aPC/PC ratio	6h Crystalloid (Liters)	2.2	.155 - 4.2	.035

aPC/PC ratio: Ratio between plasma levels of activated protein C and protein C zymogen at the admission to the hospital.

Table 3: Logistic regression of protein C system effects on outcome.

Predictor	Dependent	OR	CI	P
aPC/PC ratio	VAP	2.4	1.6 - 2.62	.024
aPC/PC ratio	MOF	1.585	1.0 - 2.5	.050
aPC/PC ratio	ALI	1.894	1.1 - 3.1	.01
aPC/PC ratio	Mortality	2.1	1.4 -3.3	.0007

aPC/PC ratio: Ratio between plasma levels of activated protein C and protein C zymogen at the admission to the hospital. VAP: Ventilator-associated pneumonia. MOF: Multiple organ failure. ALI: Acute lung injury.

Table 4: Activation of protein C and hospital outcome

Predictor	Dependent	B	CI	P
aPC/PC ratio	Ventilator days	5.63	3.49-7.77	<.0001
aPC/PC ratio	ICU days	5.26	3.23-7.30	<.0001
aPC/PC ratio	Hospital days	4.17	2.40-5.94	<.0001

Table 5: Relationship between depletion of protein C over time and outcome

Predictor	Dependent	OR	CI	P
12 hour PC depletion	VAP	.985	.97 - .99	.04
Balanced PC response	VAP	Referent		
Moderate Depleters	VAP	1.6	.7 - 4.1	.2
PC Depleters	VAP	2.7	1.05 – 6.8	.04

VAP: Ventilator-associated pneumonia.

Figure 1: A combination of tissue injury and shock result in prolonged prothrombin time and prolonged partial thromboplastin time.
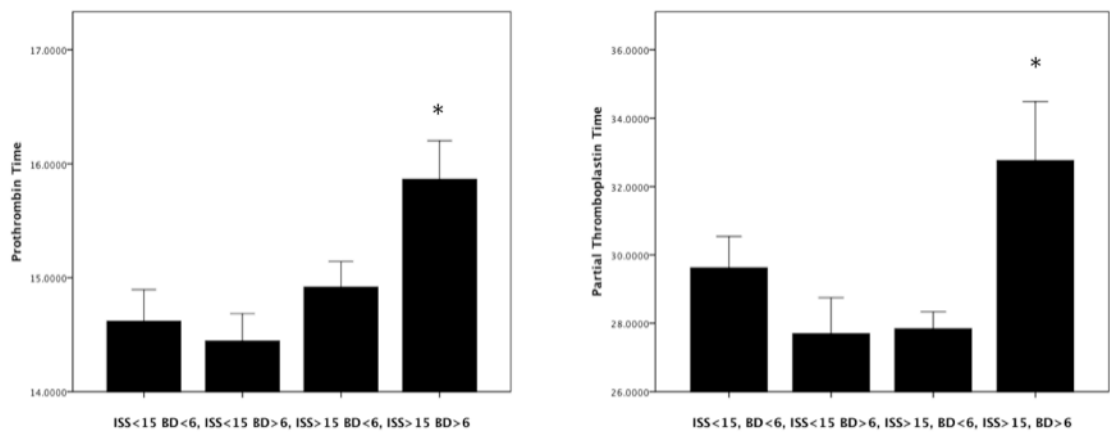
Figure 2: Tissue injury and shock result in activation of protein C and coagulopathy
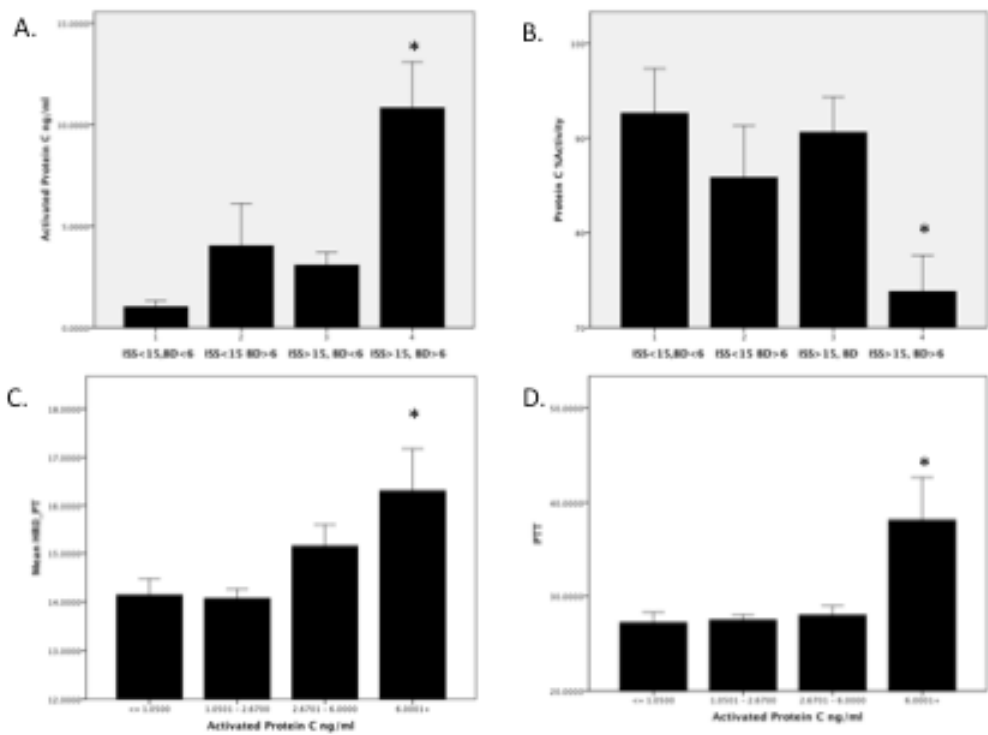
Figure 3: Activation of protein C is related to inactivation of Va and VIIIa and derepression of fibrinolysis.
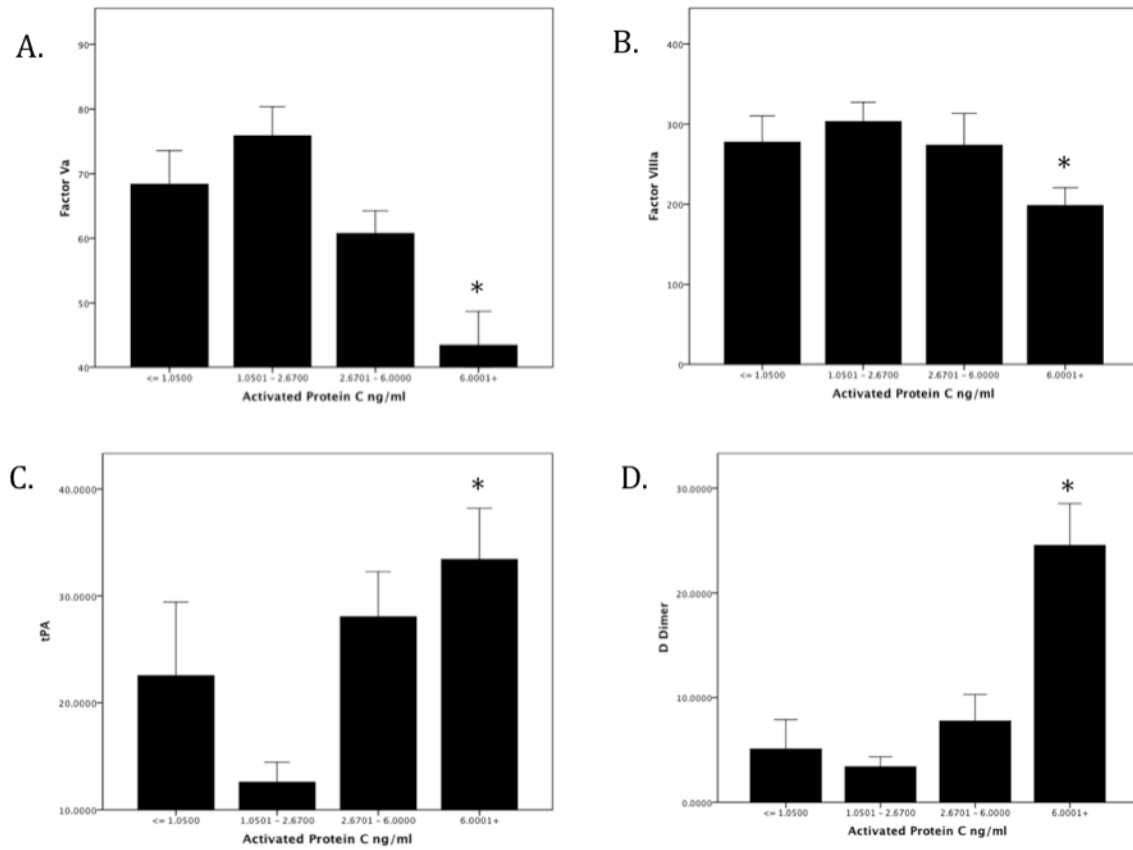
Figure 4: <mark>Depletion of PC is associated with an increased risk of VAP</mark>

Legend:
- BALANCED PC RESPONSE
- MODERATE PC DEPLETION WITH RECOVERY
- PC DEPLETION

OR VAP 2.7 p=.04
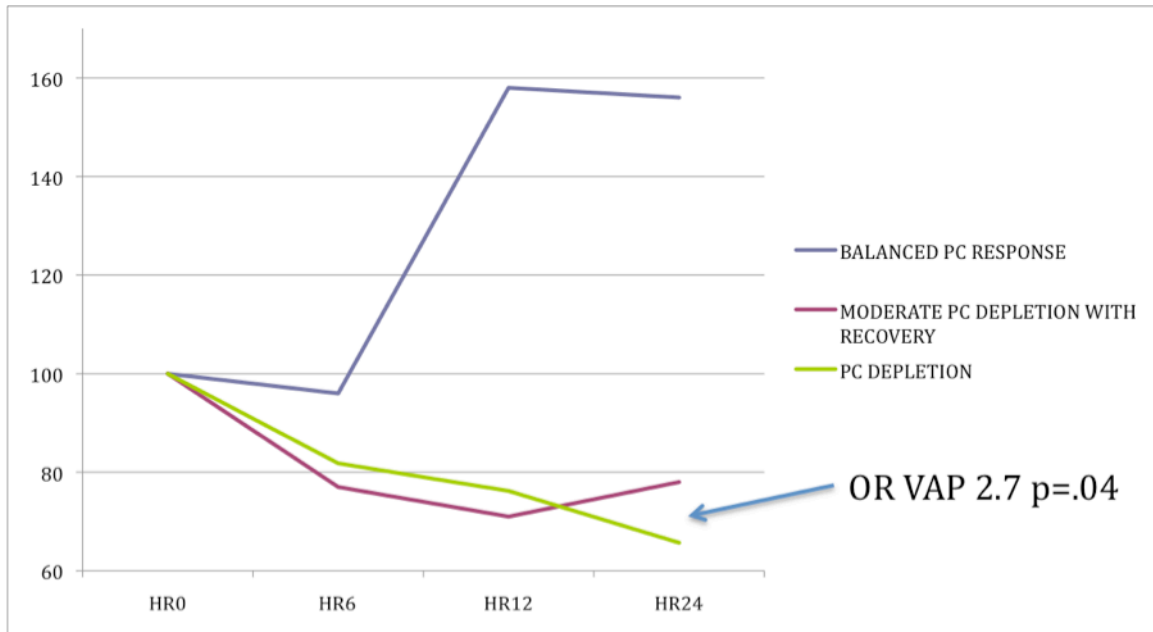
INCREASE IN ACTIVATED PROTEIN C MEDIATES ACUTE TRAUMATIC COAGULOPATHY IN MICE

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ABSTRACT—In severely injured and hypoperfused trauma patients, endogenous acute coagulopathy (EAC) is associated with an increased morbidity and mortality. Recent human data correlate this coagulopathy with activation of the protein C pathway. To examine the mechanistic role of protein C in the development of EAC, we used a mouse model of trauma and hemorrhagic shock, characterized by the combination of tissue injury and severe metabolic acidosis. Mice were subjected to one of four treatment groups: 1) C, control; 2) T, trauma (laparotomy); 3) H, hemorrhage (MAP, 35 mmHg × 60 min); 4) TH, trauma + hemorrhage. After 60 min, blood was drawn for analysis. Compared with C mice, the TH mice had a significantly elevated activated partial thromboplastin time (23.3 vs. 34.5 s) and significantly increased levels of activated protein C (aPC; 2.30 vs. 13.58 ng/mL). In contrast, T and H mice did not develop an elevated activated partial thromboplastin time or increased aPC. Selective inhibition of the anticoagulant property of aPC prevented the coagulopathy seen in response to trauma/hemorrhage (23.5 vs. 38.6 s [inhibitory vs. control monoclonal antibody]) with no impact on survival during the shock period. However, complete blockade of both the anticoagulant and cytoprotective functions of aPC caused 100% mortality within 45 min of shock, with histopathology evidence of pulmonary thrombosis and perivascular hemorrhage. These results indicate that our unique mouse model of T/H shock mimics our previous observations in trauma patients and demonstrates that EAC is mediated by the activation of the protein C pathway. In addition, the cytoprotective effect of protein C activation seems to be necessary for survival of the initial shock injury.

KEYWORDS—Trauma, shock, hemorrhage, hypoperfusion, coagulation, survival

INTRODUCTION

Trauma remains the leading cause of death and disability in adults, eclipsing ischemic heart disease, cerebrovascular disease and human immunodeficiency virus/AIDS (1). Worldwide, one in seven deaths is due to injury, and this is expected to rise to one in five in the next 15 years, despite continuing advances in resuscitation, trauma surgery, and critical care (2). Hemorrhage is responsible for 40% of early trauma deaths, and efforts to control hemorrhage and restore circulatory homeostasis form the core of the therapeutic approach to traumatic injuries (3).

Perturbations in blood coagulation are common after major trauma and are associated with poor outcomes (1, 4). Classically, coagulopathy associated with trauma is thought to be due to the consumption of coagulation factors, acidosis, dilution from intravenous blood and fluid therapy, and hypothermia (5). This coagulopathy can be described as systemic acquired coagulopathy (SAC) (6). The abnormalities associated with SAC have been partially characterized in animal models and extensive clinical human research (7–9). In addition, there is extensive literature exploring the ideal resuscitative protocol and treatment of SAC (5, 7, 10).

More recently, it has been recognized that some trauma patients present with an early coagulopathy that is physiologically and mechanistically distinct from SAC. Two recent studies have identified an acute traumatic coagulopathy, present on arrival in the emergency department, in 25% of patients with major trauma (11, 12). This posttraumatic endogenous acute coagulopathy (EAC) is associated with higher transfusion requirements, a greater incidence of multiple organ dysfunction syndrome, longer intensive care unit and hospital stays, and a 4-fold increased risk of mortality compared with those with normal coagulation (11, 12). In examining the mechanism for this EAC, we reported that the combination of traumatic injury and hypoperfusion (shock) resulted in a coagulopathy that was associated with a reduction in protein C (PC) levels (13).

Protein C is a plasma serine protease that is activated through a thrombin-dependent reaction also involving thrombomodulin and the endothelial protein C receptor (14). Once activated, aPC exerts its anticoagulant effects by irreversibly inactivating factors Va and VIIIa (14). In addition, aPC has anticoagulant activity through its derepression of fibrinolysis

by directly inhibiting plasminogen activator inhibitor 1 (15). Activated protein C (aPC) also acts via the cell surface receptor, protease-activated receptor 1 (PAR-1) to produce several cytoprotective effects (16). These effects include anti-inflammatory properties, antiapoptotic activity, and protection of endothelial barrier function (17–19).

Several mouse models have been used to study the effects of trauma and hemorrhagic shock on the following: survival, organ perfusion, immune response, inflammation, injury to lung and liver, and the impact of sex on the response to trauma and hemorrhage (20–25). In addition, although other animal models have examined the coagulopathy associated with the classic mechanisms of hypothermia, dilution, factor consumption, and acidosis, no published models have been adapted to study EAC associated with trauma (7–9, 26). Therefore, the first aim of the present study was to optimize a preexisting mouse model of trauma and hemorrhagic shock (20) to develop the first animal model of EAC that is seen after tissue injury and hypoperfusion in trauma patients. Second, we set out to use this model to examine the hypothesis drawn from our correlative human data that activation of PC is the primary mechanism responsible for the development of posttraumatic EAC. Finally, using our model, we also questioned whether this activation of PC could be a physiologic response to severe trauma/hemorrhage necessary for survival of the initial shock injury.

MATERIALS AND METHODS

Mouse model of trauma-hemorrhage and acute traumatic coagulopathy

Protocol was developed, based in principle, on the mouse model of traumatic shock extensively published by several investigators (20, 22, 27). The experiments were conducted in accordance with National Institutes of Health (Bethesda, Md) guidelines and approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

Male C57BL/6J mice 8 to 10 weeks old (Jackson Laboratory, West, Sacramento, Calif) were used in all experiments. Animals were allowed water and food *ad libitum* and given at least 24 h to acclimate to the housing facility. The mice were anesthetized with isoflurane 1.2% in air:O$_2$ at 1:0.5 L/min. The animals were then secured with plastic tape in a supine position on a firm plastic board. A lubricated rectal temperature probe was inserted and continuously monitored. A heat lamp was used to maintain core body

temperature between 36 and 37°C throughout the experiment. As a model of soft tissue trauma, a sterile 2-cm midline laparotomy was performed. After inspection of underlying organs to verify absence of damage from the laparotomy, the wound was closed with a single layer of sterile wound clips (Reflex 7-mm wound clips; Braintree Scientific, Braintree, Mass). Then, the left femoral artery and right femoral vein were cannulated with PE-10 tubing preflushed with isotonic sodium chloride solution. After the laparotomy and vessel cannulations, all incisions were bathed with lidocaine 1% for analgesia, and the isoflurane was discontinued to allow emergence from general anesthesia.

MAP was monitored by attaching the left femoral arterial catheter to a pressure transducer (TSD104A; Biopac Systems, Goleta, Calif) and amplifier (MP1004-CE; Biopac Systems). The transducer output was analyzed using AcqKnowledge Software (Biopac Systems) with the arterial waveform continuously displayed. Upon emergence from anesthesia, baseline MAP greater than 90 mmHg was confirmed before initiation of the shock period. Shock was induced by withdrawing blood into a 1-mL syringe containing 3.2% sodium citrate via the left femoral arterial catheter 10 min after discontinuation of anesthesia at a rate of 0.2 mL/min. Arterial pressure was measured every minute during hemorrhage until the target MAP (35 mmHg) was achieved. This target MAP (35 ± 5 mmHg) was maintained over the course of the 60-min shock period by repeatedly removing additional aliquots of blood. Temperature, MAP, and respiratory rate were recorded every 5 min. Tachypnea seen in the trauma + hemorrhage (TH) and hemorrhage alone (H) groups at the end of the shock period was used as a reassuring surrogate marker for metabolic acidosis and systemic hypoperfusion, although no interventions were protocolized to target a specific respiratory rate (Fig. 1). Control (C), trauma (T), and hemorrhagic shock (H) mice underwent the same anesthetic, catheter placement, and 60-min period of board stress. Hemorrhagic mice underwent hemorrhagic shock only (no laparotomy), the T mice underwent laparotomy only (no hemorrhagic shock), and the C mice did not receive either the laparotomy or the hemorrhagic shock. Throughout the shock period, isotonic sodium chloride solution was used to gently flush the catheters to maintain catheter patency. Total volume of isotonic sodium chloride solution (crystalloid) administered to the animals was minimal and is presented in the Table 1.

After 60 min of shock (H, TH) or board stress (C, T), mice were quickly euthanized with an overdose of isoflurane. Blood was drawn via inferior vena cava (IVC) puncture into a 1-mL syringe containing either 3.2% sodium citrate (for analysis of lactate, hemoglobin, activated partial thromboplastin time [aPTT], aPC) or heparin sulfate (for blood gas analysis and correlative lactate measurement). To preserve the aPC for later analysis, an aliquot of citrated whole blood was removed and mixed with the reversible protease inhibitor benzamidine (final concentration, 1 mM). All citrated blood samples were centrifuged at 4,000 rpm × 10 min, the supernatant removed and centrifuged at 13,000 rpm × 10 min to remove remaining cells and platelets. The final plasma supernatant was then removed and stored at −80°C until the time of analysis. After euthanasia and IVC blood draw, the lungs were removed and formalin-fixed. Organs were subsequently paraffin-embedded, sectioned, and hematoxylin and eosin stained. Blinded pathological assessment was performed by trained pathologists from the Department of Pathology at University of California at San Francisco.
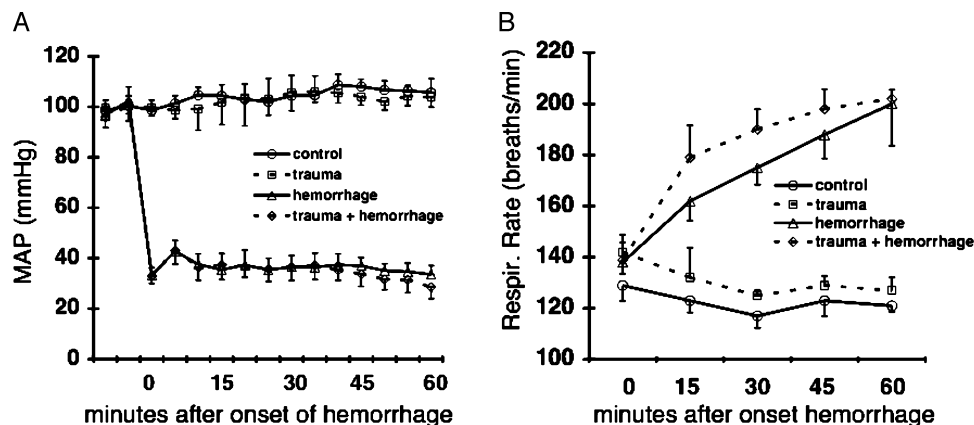

FIG. 1. **Hemodynamic and respiratory responses to hemorrhagic shock**. A–B, Changes in MAP (A) and respiratory rate before and during hemorrhagic shock (B). Control mice underwent catheter placement only. Trauma mice received 2-cm midline laparotomy and closure, followed by catheter placement. Hemorrhage mice underwent catheter placement, followed by hemorrhagic shock (blood withdrawal(s) necessary to maintain MAP = 35 ± 5 mmHg for 60 min). Trauma + hemorrhage mice received 2-cm midline laparotomy and closure, catheter placement, and hemorrhagic shock. Data are expressed as mean ± SD (n = 10 per group).

Table 1. **Characteristics of the model and experimental groups**

	Control	Trauma	Hemorrhage	Trauma + hemorrhage	TH mAb 1761	TH mAb 1591	TH mAb 1609
Weight, g	25.1 ± 1.1	24.7 ± 1.7	23.7 ± 1.7	24.2 ± 2.8	25.3 ± 1.6	24.9 ± 1.2	24.8 ± 1.3
Hemorrhage, %ETBV	0	0	40.5 ± 1.8	37.0 ± 3.5	34.2 ± 5.1	35.1 ± 2.5	30.8 ± 3.7
Crystalloid, %ETBV	4.0 ± 0.9	6.1 ± 1.7	6.0 ± 1.5	6.2 ± 1.9	7.9 ± 2.2	8.0 ± 1.3	7.0 ± 1.8
Surgery time, min	26 ± 7	32 ± 3	23 ± 5	33 ± 10	39 ± 6	36 ± 7	35 ± 4
Hgb: end-shock, g/dL	13.1 ± 0.6	13.5 ± 0.7	9.1 ± 0.5	10.1 ± 1.0	9.2 ± 1.3	10.1 ± 0.9	10.9 ± 1.5
Lactate: end-shock, mM	4.6 ± 0.7	6.5 ± 0.8	10.7 ± 3.6	10.7 ± 2.1	9.7 ± 1.8	10.4 ± 3.3	13.2 ± 4.6

Groups treated as described in the "Materials and Methods" section. Hemorrhage is total blood volume removed over course of 60-min shock period. Crystalloid is total crystalloid volume administered during surgery and shock period. ETBV = weight (g) × 0.077 mL/g. Hemoglobin (Hgb) and lactate values are measured at the end of 60-min shock period.

Reagents

Monoclonal antibodies (mAbs) blocking specific functions of PC were generously provided by Charles Esmon, Ph.D. (Oklahoma Medical Research Foundation, Oklahoma City, Okla). Monoclonal antibody 1609 inhibits binding to cell surfaces and thus blocks both the anticoagulant and cytoprotective functions of PC. Monoclonal antibody 1591 selectively inhibits only the anticoagulant function of aPC but allows binding to cell surfaces and thereby cellular signaling (manuscript in preparation). Monoclonal antibody 1761 is an immunoglobulin G1 rat-antimouse isotype control antibody. Animals pretreated with mAbs received 10 mg/kg of mAb via the right femoral venous catheter 10 min before initiation of hemorrhagic shock. Gentle aspiration of venous blood after administration of mAb confirmed successful intravenous injection.

Assays

Activated partial thromboplastin time was performed on mouse plasma using the STACompact coagulation analyzer (Diagnostica Stago, Inc., Parsippany, NJ). Hemoglobin values were measured on whole blood samples using the B-Hemoglobin Photometer (HemoCue AB, Angelholm, Sweden).

Blood gas measurements were performed immediately on heparinized whole blood using an Osmetech OPTI CCA blood gas analyzer (OPTI Medical, Inc. Roswell, Ga). Lactate measurements were also performed immediately on all whole blood samples using the Accutrend Lactate analyzer (Roche Diagnostics, Indianapolis, Ind).

Activated protein C levels were measured in our laboratory using an assay specific for the activated form of PC developed in the laboratory of Esmon (28). Briefly, a 96-well vinyl plate was coated overnight at 4°C with monoclonal antibody AMGDPC 1587 at 5 μg/mL in plating buffer (0.02 M Tris, 0.1 M NaCl, pH 7.5). Plates were then blocked with blocking buffer (0.02 M Tris, 0.1 M NaCl, 1% bovine serum albumin, pH 7.5). Standard dilutions of mouse aPC and mouse plasma samples were loaded onto the plate and incubated at room temperature for 2 h on a 150-rpm rocker. Plates were washed with wash buffer (0.02 M Tris, 0.1 M NaCl, 0.05% Tween, pH 7.5). Spectrazyme PCA at 1 mM in plating buffer was added and incubated at 37°C for 24 h. Absorbance was measured at 405 nm with sample concentration derived from standard curve.

Statistical analysis

All data expressed as mean ± SD. Because of the sample sizes of the experimental groups, all comparison of continuous variables between groups was done using a nonparametric Kruskal-Wallis one-way ANOVA. A Mann-Whitney test was then used as a post hoc test for intergroup comparison.

Survival data were analyzed using a Kaplan-Meier analysis for the curve with a log-rank test for significance. Significance is defined as a P value less than 0.05.

RESULTS

Mouse model of acute traumatic coagulopathy

Systemic physiology—As shown in Figure 1, MAP was successfully held within the goal range of 35 ± 5 mmHg for the 60-min shock period in both the H and TH groups. In contrast, the C and T groups remained normotensive throughout the 60-min period. To maintain a MAP = 35 ± 5 mmHg, animals undergoing H alone required 5.2 ± 1.1 (mean ± SD) blood withdrawals ranging in volume from 0.03 to 0.10 mL over the course of the 60-min shock period, whereas those in the TH group required 4.9 ± 0.7 withdrawals with volumes between 0.03 and 0.09 mL (P = not significant [NS]). Total hemorrhage volume, as defined as percentage of estimated total blood volume (ETBV), was not significantly different between the H and TH groups (Table 1). Interestingly, the H and TH groups each developed a progressive tachypnea over the course of the shock period, whereas the C and T groups sustained a normal respiratory rate (Fig. 1).

Hypoperfusion—The H and TH groups each developed a significant and severe metabolic acidosis after 60 min of hemorrhagic shock. These groups each manifested significantly decreased pH, decreased base excess, and increased lactate when compared with the C and T groups (Fig. 2, A–C). In this model, clinically significant base excess is defined as less than −6 mM, which corresponds to a plasma lactate value of 7.0 mM (Fig. 2D). Measurement of plasma lactate values in each individual animal allowed direct confirmation of a significant hypoperfusion injury in all experiments.
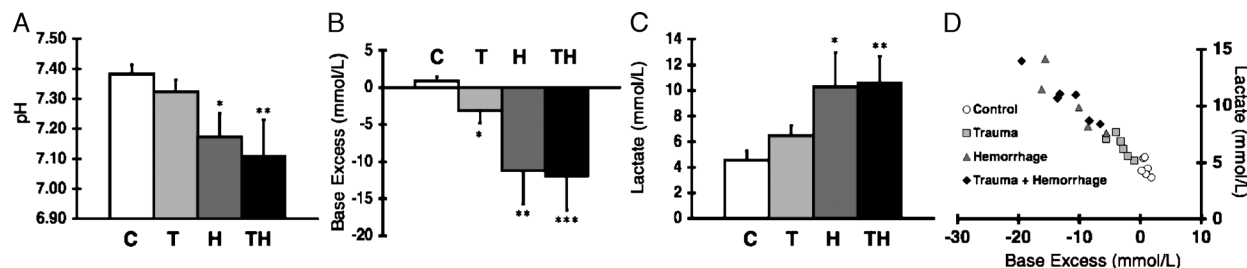

Fig. 2. **Systemic markers of hypoperfusion in mice after TH shock**. A–D, Mice underwent either TH, H, T, or C (no trauma, no shock). Blood was drawn after 60 min of hemorrhagic shock via IVC puncture. Data expressed as mean ± SD (n = 5 per group). A, *P < 0.02 (C vs. H, T vs. H); **P < 0.02 (C vs. TH, T vs. TH). B, *P < 0.01 (C vs. T); **P < 0.01 (C vs. H, T vs. H); ***P < 0.01 (C vs. TH, T vs. TH). C, *P < 0.01 (C vs. H, T vs. H); **P < 0.01 (C vs. TH, T vs. TH).
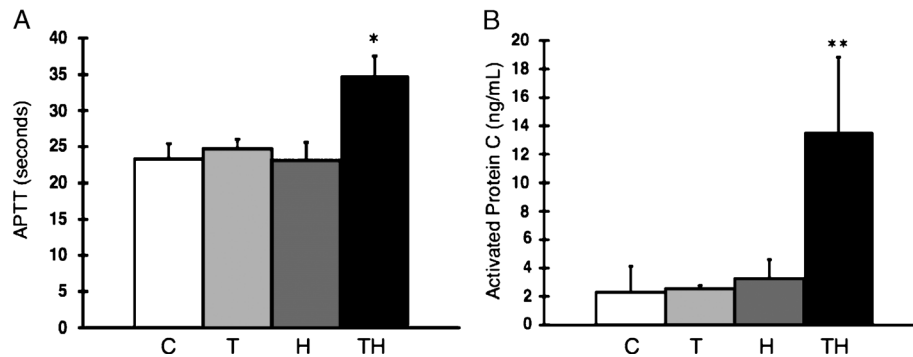
FIG. 3. **Trauma + hemorrhagic shock is associated with increases in aPTT (in seconds) and aPC (in nanograms per milliliter) in mice**. A and B, Mice underwent either TH, H, T, or C (no trauma, no shock). Activated partial thromboplastin time (in seconds) and aPC (ng/mL) levels were measured after 60 min of hemorrhagic shock. Data were expressed as mean ± SD. C (n = 6), T (n = 6), H (n = 10), TH (n = 10). A, *P = 0.02 (C vs. TH, T vs. TH, H vs. TH). B, **P < 0.02 (C vs. TH, T vs. TH, H vs. TH).

Acute traumatic coagulopathy—As demonstrated in Figure 3, trauma + hemorrhagic shock is associated with increases in aPTT and aPC levels, similar to those seen in coagulopathic human trauma patients. In this model, the development of coagulopathy requires traumatic injury and a significant hypoperfusion injury because the presence of T or H was not sufficient to increase aPTT or aPC levels. Interestingly, as compared with C mice, those few TH mice with a lactate value less than 7 mM did not have elevated aPTT (23.3 vs. 25.6 s; P = NS) or aPC (2.30 vs. 1.96 ng/mL; P = NS; data not shown). Classic mechanisms of SAC were minimized throughout the experiment to isolate the mechanism(s) of EAC. Core temperature was monitored and maintained between 36 and 37°C throughout the shock period and catheter placement. In addition, dilution of coagulation factors by crystalloid administration was minimal and not significantly different between experimental groups (Table 1). Comparable relative volumes of crystalloid for a 70-kg human patient are approximately 350 mL.

Role of aPC in acute traumatic coagulopathy and traumatic shock

Inhibition of aPC anticoagulant function prevents development of acute traumatic coagulopathy—Trauma and hemorrhagic mice pretreated with mAb 1591, which selectively inhibits the anticoagulant function of aPC, do not develop an increased aPTT after traumatic shock (Fig. 4). However, TH mice pretreated with an isotype control mAb1761 develop acute traumatic coagulopathy with an elevated aPTT (Fig. 4). Total hemorrhage volume (34.2 ± 5.1 vs. 35.1 ± 2.5 %ETBV), total crystalloid volume (7.9 ± 2.2 vs. 8.0 ± 1.3 %ETBV), and end-shock lactate (9.7 ± 1.8 vs. 10.4 ± 3.3 mM) were not significantly different between the mAb1761-pretreated and mAb1591-pretreated groups, respectively (Table 1). Pretreatment of C mice with either mAb (1761 vs. 1591) had no effect on aPTT values.
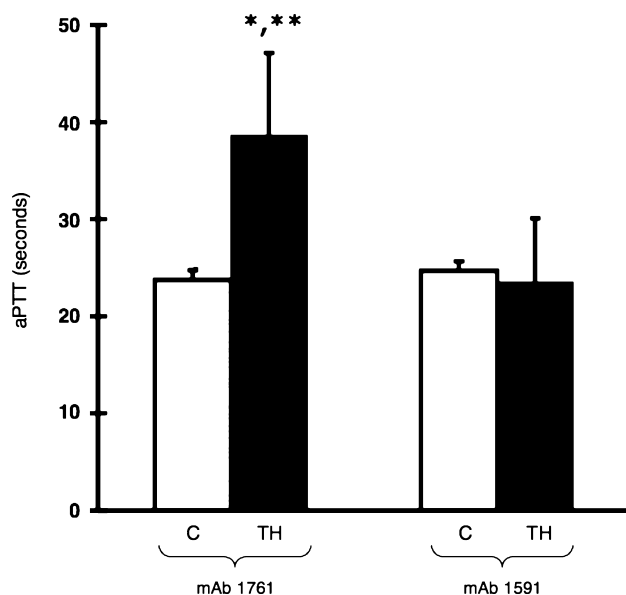

FIG. 4. **Inhibition of the anticoagulant function of PC prevents the development of acute traumatic coagulopathy in mice**. Mice were pretreated with a mAb that inhibits the anticoagulant function of PC (mAb 1591) or with an isotype control mAb (mAb 1761). After 10 min, the mice then underwent TH. Activated partial thromboplastin time values were measured after 60 min of hemorrhagic shock. Data were expressed as mean ± SD. C, 1761 (n = 5); TH, 1761 (n = 9); C, 1591 (n = 5); TH, 1591 (n = 10). *P < 0.05 (C vs. TH mAb 1761). *P < 0.05 (TH [1761] vs. TH [1591]).
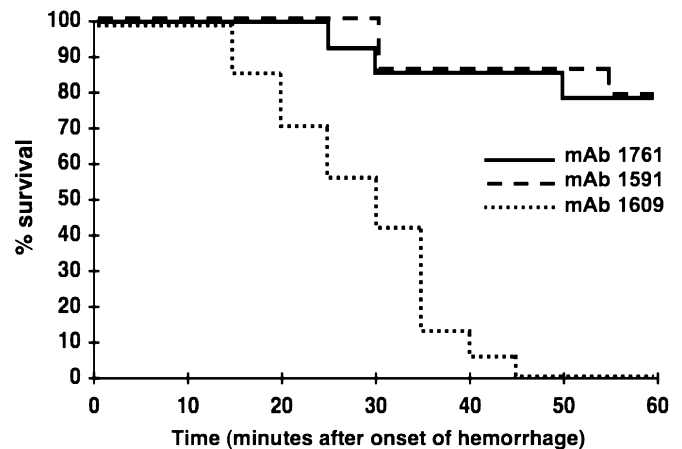

FIG. 5. **Complete inhibition of PC before TH leads to 100% mortality within 45 min of shock**. Mice were pretreated with one of three monoclonal antibodies: mAb 1609 inhibits both the cytoprotective and anticoagulant functions of PC, mAb 1591 inhibits only the anticoagulant function of PC, and mAb 1761 is an isotype control monoclonal antibody. After 10 min, the mice then underwent TH. n = 14 mice per group. All C mice (no trauma, no shock) in all treatment groups (mAbs 1761, 1591, and 1609) survived (curves are not shown).
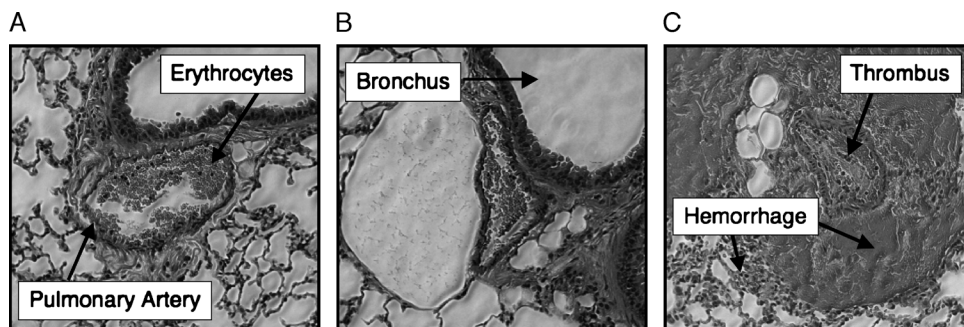
FIG. 6. **Complete inhibition of PC before TH leads to pulmonary artery thrombosis, perivascular hemorrhage, and alveolar hemorrhage in mice**. A–C, Mice were pretreated with one of three monoclonal antibodies: A, mAb 1761, an isotype control monoclonal antibody; B, mAb 1591, inhibits only the anticoagulant function of PC; C, mAb 1609, inhibits both the cytoprotective and anticoagulant functions of PC. After 10 min, the mice then underwent TH. Lungs were harvested after 60 min of hemorrhagic shock or at the time of death. Lungs were fixed, sectioned, and hematoxylin and eosin stained. Images obtained at 200× magnification. All C mice (no trauma, no shock) in all treatment groups (mAbs 1761, 1591, and 1609) had no pulmonary pathology (sections not shown).

Complete inhibition of aPC prevents survival of traumatic shock—Trauma and hemorrhagic mice pretreated with mAb1609, which completely inhibits both the anticoagulant and cytoprotective functions of PC, suffer 100% mortality within 45 min of shock (Fig. 5). In contrast, TH mice pretreated with either mAb1761 or mAb1591 have similar survival curves with 20% mortality after 60 min of shock. All C mice (no trauma, no shock) in all treatment groups (mAbs 1761, 1591, and 1609) survived the 60-min protocol (data not shown).   Histological analysis of pulmonary tissues revealed evidence of diffuse pulmonary arteriolar thrombosis as well as perivascular and alveolar hemorrhage in the TH mice pretreated with mAb 1609 (Fig. 6). Trauma and hemorrhagic mice pretreated with mAbs 1761 and 1591 did not develop any pulmonary pathology. In addition, C mice pretreated with mAbs, either 1761, 1591, or 1609, had normal pulmonary histology (not shown).

DISCUSSION

In this study, we present the development of the first animal model of posttraumatic EAC. Our group has previously shown that the combination of trauma and shock (tissue hypoperfusion) in human patients is associated with an activation of PC, early coagulopathy, and poor outcome (13). Although highly suggestive of an aPC mediated early traumatic coagulopathy, these human data were observational and correlative, requiring mechanistic confirmation. To provide mechanistic confirmation of our human data, we developed a translational mouse model of acute traumatic coagulopathy. Using this mouse model, we demonstrated that the combination of tissue injury and tissue hypoperfusion is required to produce an early traumatic coagulopathy. The coagulopathy seems to be mediated by aPC because blocking the anticoagulant activity of this enzyme prevents coagulopathy (Fig. 4).

Coagulopathy after traumatic injury has long been appreciated as an ominous complication and management challenge (29, 30). Our previously published human data showed a coagulopathy that was independent of traditional causes of posttraumatic SAC (hypothermia, dilution, consumption, or acidemia) due to an EAC (13). This EAC begins nearly immediately after trauma in the presence of shock and is

distinct from SAC. Because we were interested in elucidating the mechanism of EAC, we attempted to minimize the impact of SAC in our model to isolate the development and mechanism(s) of EAC. As such, normothermia was maintained throughout the entire protocol, and dilution of coagulation factors with crystalloid was minimal. Although acidemia is known to contribute to coagulopathy, in our study, it was not sufficient on its own to trigger a coagulopathy because the mice subjected to H developed a significant and severe acidosis but did not become coagulopathic. As a result, hypoperfusion is clearly required in conjunction with traumatic injury to manifest EAC, whereas acidemia alone likely exacerbates and perpetuates the mechanisms and development of SAC. The posttraumatic coagulopathy is likely one of multiple phenotypes beginning with EAC and transitioning to SAC over time.

Consistent with observations in human trauma patients, our mouse model of EAC requires both tissue injury and severe hypoperfusion to manifest the coagulopathy. However, consistent with clinical observation of trauma patients, we occasionally observed heterogeneity produced by the injury/shock protocol, with rare mice not manifesting a significant hypoperfusion injury despite the same degree of hypotension. As a result, we confirmed the degree of hypoperfusion injury in each animal by measuring plasma lactate levels to appropriately group those individuals with documented severe hypoperfusion. Interestingly, those few animals not manifesting a severe hypoperfusion injury did not activate PC and did not develop EAC in response to traumatic injury and hypotension. Conversely, all mice that developed a severe hypoperfusion injury had an increased aPC and coagulopathy.

Our human data suggest that consumption of PC through conversion to aPC is associated with posttraumatic EAC (unpublished data) (13). The present study provides the mechanistic proof that our interpretation of the human correlative data is correct. Furthermore, as the first animal model of EAC, these data demonstrate that the anticoagulant function of aPC is a primary mechanism responsible for the development of EAC. Selective inhibition of PC's anticoagulant function in our animal model effectively prevented the development of EAC in response to injury and hypoperfusion. This result is consistent with our published clinical observations

that activation of the PC pathway correlates with the development of EAC in injured and hypoperfused trauma patients.

Although PC is clearly activated in response to the combination of traumatic injury and hypoperfusion, the mechanism of this PC activation is, as yet, unknown. Perhaps, a threshold of inflammation, injury, and/or hypoxia is required to trigger the activation of PC. Human data suggest that the activation of complement subsequently triggers the activation of PC in response to trauma and hypoperfusion (31). *In vitro* and additional *in vivo* experiments are presently underway in our laboratory to further elucidate the mechanism of this PC activation after trauma and hypoperfusion.

Activated PC is a serine protease that exerts its anticoagulant effects by irreversibly inactivating factors Va and VIIIa and by derepressing fibrinolysis through its inhibitory actions on plasminogen activator inhibitor 1 (15). In addition to its anticoagulant effects, aPC proteolytically activates the cell surface receptor PAR-1 to produce several cytoprotective effects, including anti-inflammatory properties, antiapoptotic activity, and protection of endothelial barrier function (17–19). Several animal models suggest that these cytoprotective properties of aPC are involved in protective responses to ischemic injury in the brain, spinal cord, heart, kidney, liver and intestine, as well as conferring protection from injury in models of sepsis (32–38). In our study, we observed that selective blockade of PC's anticoagulant function effectively inhibits the development of EAC without an impact on survival of shock. However, complete blockade of both the anticoagulant and cytoprotective functions of aPC result in 100% mortality within 45 min of shock. This finding suggests that activation of PC in response to traumatic injury and severe hypoperfusion is required for acute survival during shock.

Our findings suggest a crucial role for the cytoprotective effects of aPC in the response to trauma and hypoperfusion. The mechanism(s) responsible for precipitating the pulmonary thrombosis, alveolar and perivascular hemorrhage, and subsequent respiratory arrest in the setting of complete aPC blockade are unclear. The thrombotic response might be amplified by the loss of the cytoprotective effects of the aPC, thereby leading to exposure of the procoagulant membrane surfaces required for thrombosis. Perhaps, some degree of PAR-1 and/or endothelial PC receptor binding is required to maintain endothelial homeostasis. Recent data from Toltl et al. (39) implicate the cytoprotective function of aPC in the down-regulation of increased tissue factor expression in response to LPS stimulation in monocytes. As such, complete inhibition of aPC could allow an unchecked increase in tissue factor expression/activity in response to trauma and hypoperfusion, thereby creating a disrupted and procoagulant endothelium. Perhaps, the combination of anticoagulant and cytoprotective functions of aPC is required to prevent the observed mortality. Conversely, the activation of PC in response to injury and hypoperfusion might be necessary only for the cytoprotective effects, whereas the accompanying coagulopathy mediated by the anticoagulant properties of aPC is merely a matter of complete activation of a protein with multiple functions. These questions are certainly intriguing and are the focus of additional experiments in our laboratory.

Several limitations exist with this study. First, one must always accept the inherent limitation of using a mouse model to draw conclusions on human disease. Use of a larger mammalian model may be more applicable to human processes, but the ability to use genetically engineered mice in future experiments makes development of this model particularly useful. Second, whereas our present study focuses on the impact of the PC system on EAC, other anticoagulant pathways such as antithrombin III or tissue factor pathway inhibitor may also be involved. Because of our supportive human clinical data implicating PC in EAC and the extremely small volumes of plasma acquired from our mouse model, we chose to focus our study on the role of aPC in EAC. Finally, whereas our results suggest that the cytoprotective properties of aPC are necessary for survival of shock, this interpretation is limited by the absence of selective blockade of the cytoprotective functions of PC. Unfortunately, such inhibitory antibodies do not exist, and blockade of the PAR-1 receptor, although inhibiting the cytoprotective properties conferred by aPC binding, would also inhibit the binding of other PAR-1 ligands, including thrombin.

Most trauma patients die secondary to ongoing hemorrhage shortly after injury (3). Concurrent coagulopathy can exacerbate this hemorrhage and make surgical repair of damaged structures considerably more difficult. Although present treatment and resuscitative strategies focus on minimizing the development of SAC, the presence of EAC upon admission to the emergency department places trauma surgeons, anesthesiologists, and patients at an early disadvantage and demands additional treatment strategies focused on the mechanisms of EAC. As such, our study provides early evidence that selective blockade of the anticoagulant function of aPC may reduce or eliminate EAC without negatively impacting survival during traumatic injury and hypoperfusion. Specific treatment directed at EAC via blockade of aPC anticoagulant function could potentially result in less coagulopathic (medical) hemorrhage, thereby facilitating surgical control of traumatic hemorrhage and minimizing resuscitative interventions that could precipitate or exacerbate SAC. Most importantly, early control of coagulopathy and hemorrhage could result in decreased mortality secondary to hemorrhage in trauma patients. Nevertheless, additional investigation is required to examine the long-term impact of selective anticoagulant aPC blockade both during and after resuscitation.
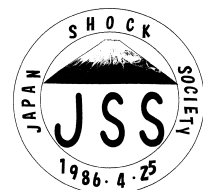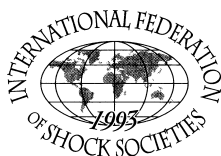
ACKNOWLEDGMENTS

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Relationship between Injury Severity Score, thrombin production and complement after severe trauma and hemorrhage in humans
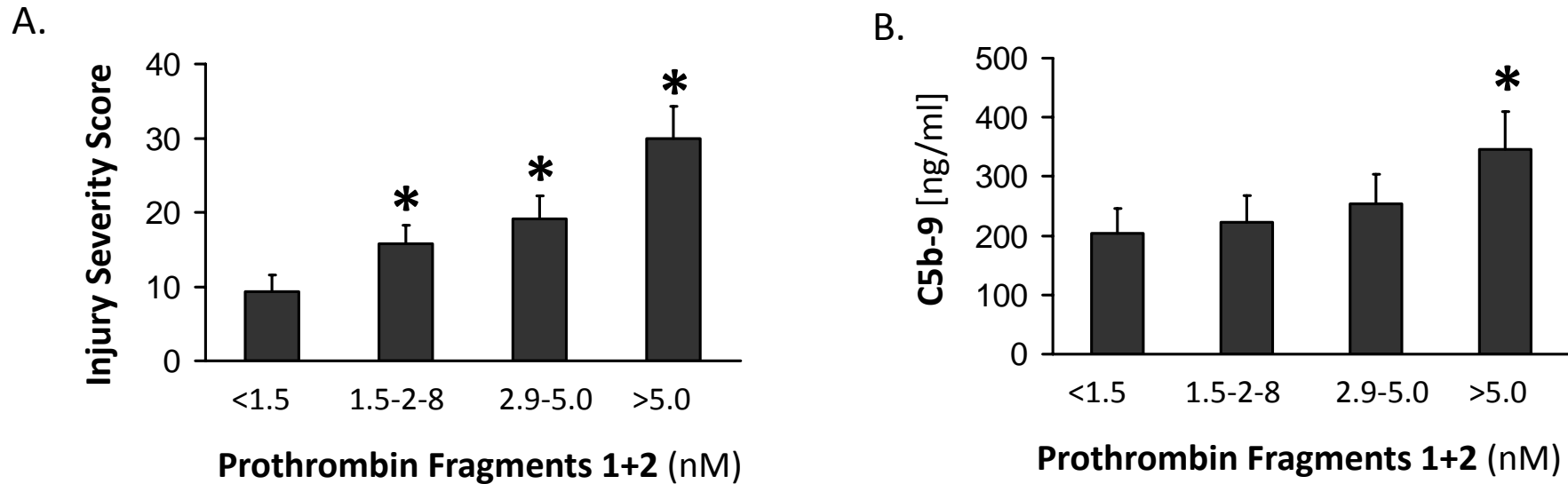
A.

B.

Figure 1.

Complement activation is associated with coagulation abnormalities, increased release of soluble thrombomodulin and activation of the protein C pathway after severe trauma and hemorrhage in humans
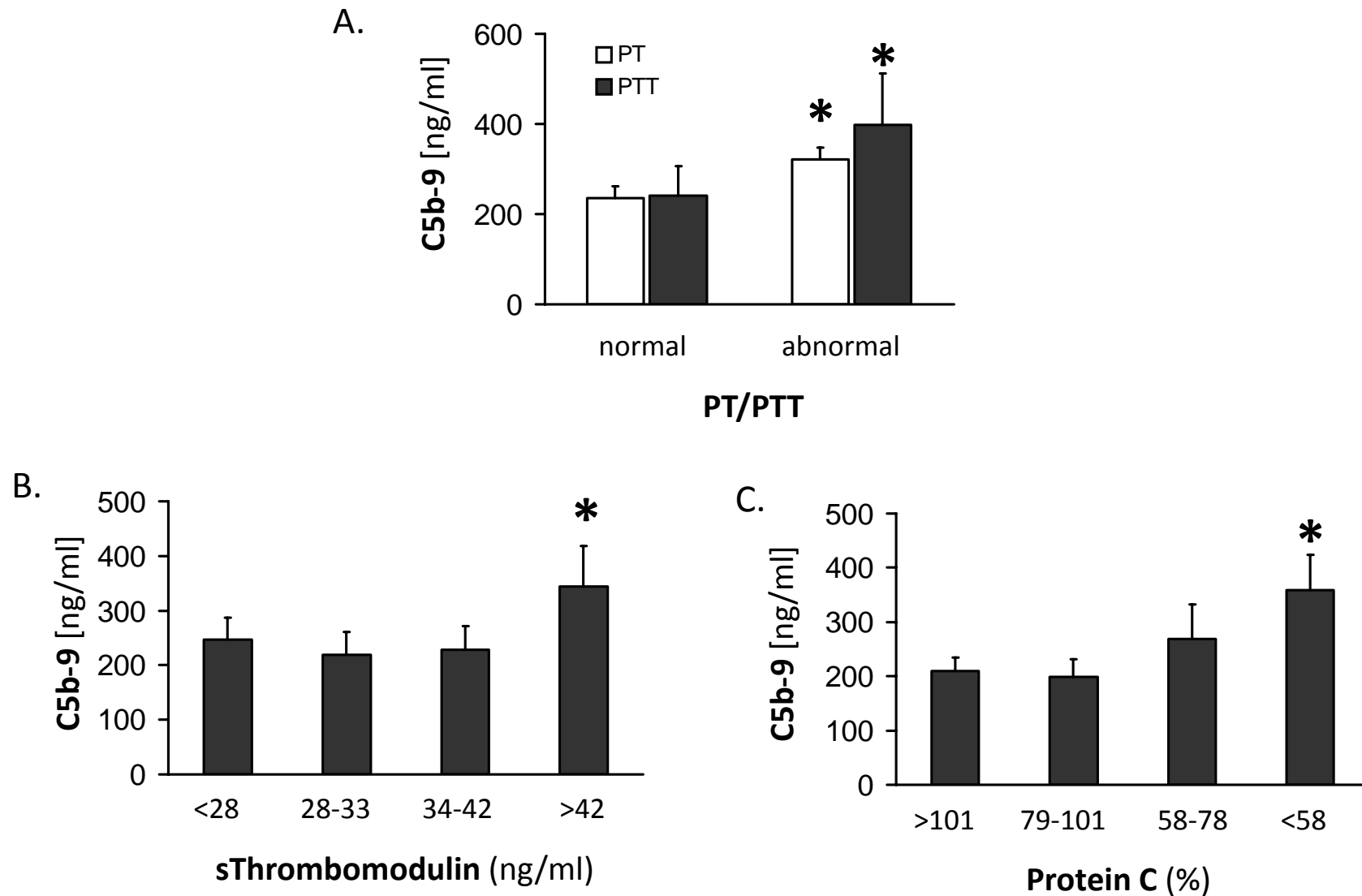

Figure 2.

Complement activation is associated with increased fibrinolysis after severe trauma and hemorrhage in humans
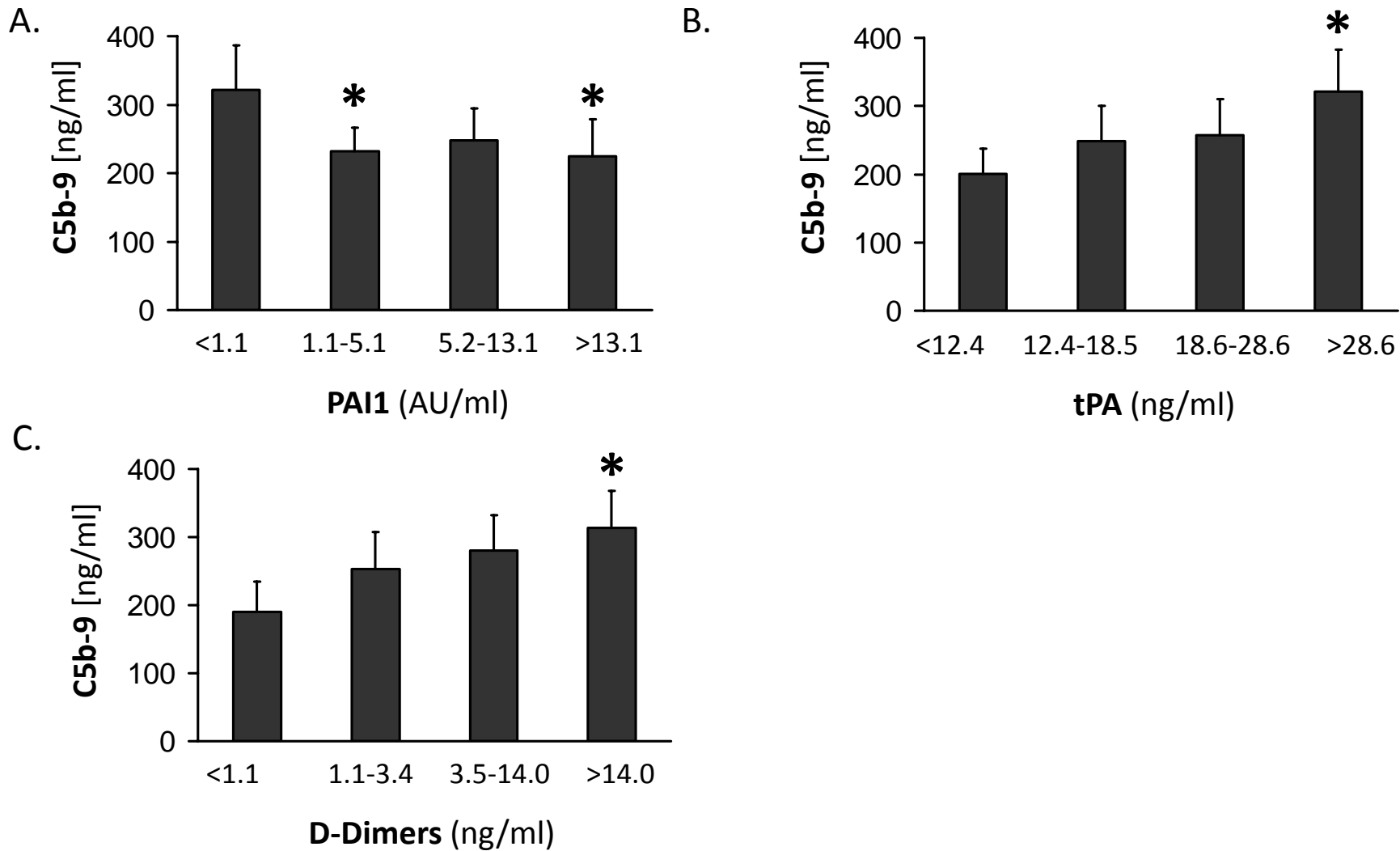
A.

B.

C.

Figure 3.

Complement activate the protein C pathway in mice and MBL null mice do not activate protein C after trauma and hemorrhage

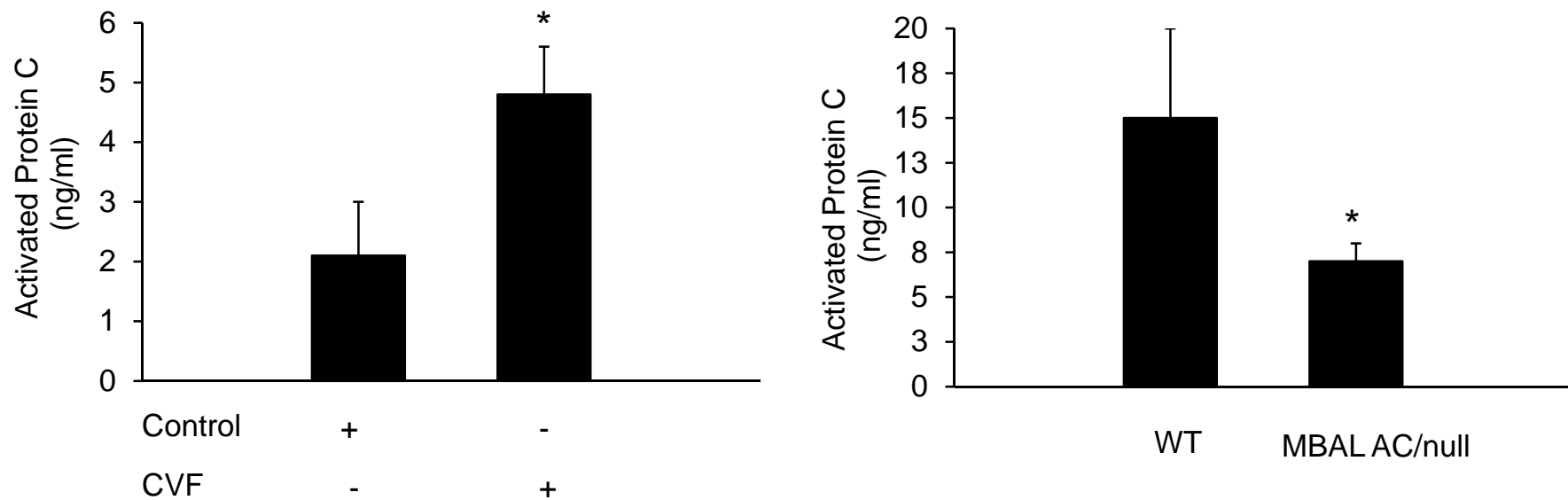**Figure 4.**